RECURRING HYBRID FORMATION IN A POPULATION OF *POLYSTICHUM ×POTTERI*: EVIDENCE FROM CHLOROPLAST DNA COMPARISONS[1]

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ABSTRACT

The origins of a population of the hybrid fern *Polystichum ×potteri* (*P. acrostichoides* × *P. braunii*) were explored through an examination of restriction fragment length polymorphisms in chloroplast DNA. We demonstrate that the hybrid individuals in this population each had one of the two possible parental chloroplast genomes. This result documented recurrent hybridization in the population, whereas isozymes and morphological data did not. As expected, the hybrids combined the length polymorphisms in ribosomal DNA detected in each parent. We suggest that inferences from chloroplast DNA data about evolution in lineages rich in hybrid species can be biased by inheritance of two chloroplast genomes in three or more different descendants of a paleopolyploid species.

Plant organelle inheritance has been the focus of many investigations. The vast majority of published reports has examined reciprocal hybrids produced by crossing (Gillham, 1978) or somatic hybrids produced by protoplast fusion (Pelletier, 1986), but some studies have used natural hybrids (Palmer et al., 1983). Patterns of chloroplast inheritance were first traced by using white and green plastids as parental markers; later they were traced using specific chloroplast gene products (Gillham, 1978). More recently, chloroplast DNA fragment profiles produced by digestion with restriction endonucleases (e.g., Conde et al., 1979; Hachtel, 1980) have proven valuable as genetic markers of chloroplasts.

Surprisingly little has been reported about the inheritance of organelles in ferns. Kirk & Tilney-Bassett (1978) cited only one study: the chloroplasts of *Phyllitis scolopendrium* (L.) Newm. (as *Scolopendrium vulgare* Sm.) were found to be inherited biparentally. However, in mature plants of the rare fern hybrid *Osmunda ×ruggii* R. Tryon (Tryon, 1940; Wagner et al., 1978), the chloroplast genome of only one parent was found (Stein, 1985). Hence, the pattern of chloroplast DNA inheritance in ferns warrants further investigation.

Interspecific hybrid plants may be difficult to detect by their morphology (Doyle & Doyle, 1988), or the parentage of apparent hybrids may be uncertain (Doyle et al., 1985; Hilu, 1988; Yatskievych et al., 1988). Evidence from molecular biology has been helpful in resolving these difficulties. Electrofocusing of the small subunit of the enzyme ribulose-1,5-bisphosphate carboxylase, a nuclear DNA encoded polypeptide, has identified parents of tobacco hybrids, and examination of the large subunit (which is chloroplast DNA encoded) has determined maternal parentage (Wildman, 1983). Hybrids have also been documented by demonstrating summation of two allozymes, each fixed in one of the parent species (Werth et al., 1985a). The combination of diagnostic lengths of ribosomal DNA fragments from each parent in an intergeneric hybrid has been used as evidence for hybridity (Doyle et al., 1985). This approach was particularly helpful in the detection of *Claytonia* hybrids, where morphology is variable (Doyle & Doyle, 1988). In addition, restriction site mutations in

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chloroplast DNA have identified one parent of a hybrid in a number of studies (e.g., Palmer et al., 1983; Hilu, 1988; Yatskievych et al., 1988).

Allopolyploidy is a prevalent mode of hybrid speciation in plants. Klekowski & Baker (1966) documented the frequent occurrence of high chromosome numbers in the homosporous ferns. Recent estimates suggest that 95% of pteridophytes are polyploid and that the majority of these are allopolyploids (Grant, 1977). Although some of these reported polyploids act as genetic diploids as measured by isozyme analysis (Haufler & Soltis, 1986; Wolf et al., 1987), numerous allopolyploid taxa have been documented by a variety of approaches (Wagner & Wagner, 1980; Barrington et al., 1989). One of the best-studied fern genera in which allopolyploidy has played an important role in speciation of the group is the Appalachian *Asplenium* complex. Morphological analysis, chromosome number and pairing behavior (Wagner, 1954), flavonoid studies (Smith & Levin, 1963), and isozyme analysis (Werth et al., 1985a) have all been used to demonstrate the allopolyploid nature of members of this genus. In this complex, recurrent origins of allopolyploidy have been demonstrated by Werth et al. (1985b). Their isozyme analysis revealed several different fixed heterozygotes, strongly suggesting that the allopolyploids have formed several times.

We have examined chloroplast DNA (cpDNA) and ribosomal DNA (rDNA) of members of a population of *Polystichum* ×*potteri* Barrington, which is the triploid hybrid between *P. acrostichoides* (L.) Roth (a diploid, $n = 41$) and *P. braunii* (Spenner) Fée (an allotetraploid, $n = 82$) (Barrington, 1986). This fern population was studied to address several questions: 1) Are chloroplasts inherited uniparentally in *Polystichum* species? 2) Does a population of hybrids have cryptic variation with respect to its chloroplasts? If both chloroplast genomes can be demonstrated in the population but in different hybrid individuals, then recurrent hybrid formation would be demonstrated. 3) Are there restriction fragment polymorphisms in the ribosomal DNA of the parents, and are these different fragments both present in the hybrid offspring as would be expected from the combining of two different nuclei? 4) Do differing cytoplasms alter the morphology of hybrids with the same nuclear genome?

In this study we show that chloroplasts appear to be inherited uniparentally in *Polystichum* and that recurrent hybrid formation can be documented by the presence of two chloroplast genomes in different individuals of a hybrid population. The hybrids also show the expected additivity of restriction fragment length polymorphisms in their ribosomal DNA. No effect of cytoplasmic inheritance on morphology of the hybrids was detected.

Materials and Methods

Leaves of individuals of *Polystichum acrostichoides*, *P. braunii*, and four of their hybrids, *P.* ×*potteri*, were gathered from the talus below the limestone-rich outcrops of Barnard Gulf, Barnard, Windsor Co., Vermont, in July 1984. There, ca. 150 hybrid individuals are distributed over at least 0.4 km of east-facing slope from the foot to near the rim of the small steep-sided valley. The hybrid individuals included in this study, *Barrington 1091*, *1094*, *1097*, and *1098*, were widely separated (at least 2 m apart). They were all morphologically documented as hybrids in earlier work (Barrington, 1986). At the same time, *Barrington 1094* was documented cytologically as triploid. The irregular meiotic pairing he observed is typical of a hybrid between an allotetraploid and a nonancestral diploid. Collections of leaves from the two parents from the same site provided the material used to document the progenitor species in this study.

Chloroplasts and nuclei were isolated from the leaves as described by Palmer & Stein (1982) and Palmer (1986). Briefly, the fronds were disrupted in a large volume-to-tissue ratio (450 ml to 25–40 g of tissue) of an isolation buffer containing polyethylene glycol (PEG; M.W. 3,500) using first a blender and then a Polytron. Unbroken cells and other cellular debris were removed by filtration through a sandwich of two layers of cheesecloth and one layer of Miracloth. Nuclei and chloroplasts were collected by centrifugation in a GSA rotor at 2,500 RPM and washed with a buffer containing PEG. The re-collected organelles were suspended in a small volume of wash buffer and applied to sucrose gradients. The gradients were prepared by layering 30% on 50% sucrose. Both steps contained PEG 6000, and the gradients were allowed to diffuse overnight at 4°C before use. Centrifugation at 25,000 RPM for one hour using an SW 27 rotor separated the chloroplasts, which banded at the gradient-step interface, from the nuclei, which pelleted. Both organelles were recovered, lysed, and the DNAs were prepared in CsCl-ethidium bromide gradients. After removal of the CsCl and the ethidium bromide, the nuclear DNAs were extracted with tris-buffered phenol and ether and exhaustively dialyzed.

Chloroplast and nuclear DNAs were digested with restriction endonucleases according to the

manufacturer's instructions. For the rDNA analysis the following eight enzymes were used: *Apa* I, *Bam*H I, *Bgl* I, *Bst*E II, *Eco*R I, *Hinc* II, *Sac* I, and *Xho* I. *Hind* III and *Pst* I were used to digest the cpDNAs. Our methods for electrophoresis, blotting, and Southern hybridizations are described in Yatskievych et al. (1988), Palmer (1986), and Palmer & Stein (1982). The cloned ribosomal DNA used was pHA1 (Jorgensen et al., 1987), which contains an entire repeat unit from pea; it was kindly provided by R. Cuellar.

Results and Discussion

Chloroplast DNAs from *Polystichum acrostichoides*, *P. braunii*, and the four hybrids were digested with *Hind* III and the resultant fragments separated by gel electrophoresis (Fig. 1). It is clear that the chloroplast DNA fragments of the two parental species are very similar, yet the chloroplast DNA of *P. braunii* differs from that of *P. acrostichoides* in having a band of 11.5 kb, and *P. acrostichoides* has a band of about 9.4 kb, which is lacking in *P. braunii*. The 9.4 kb fragment was most likely produced by an additional *Hind* III site in the 11.5 kb fragment, but the presence of the other fragment (which is likely to be 2.1 kb) could not be verified in this gel. Nonetheless, the two large fragments provide a clear criterion to distinguish the parental genomes. The fragment profiles reveal that two of the hybrids inherited the *P. braunii* chloroplast genome, whereas the other two contained the *P. acrostichoides* chloroplast DNA. *Pst* I digests demonstrated the same pattern of inheritance of parental cpDNA genomes in the hybrids.

Since only one parental chloroplast genome is found in each of the hybrids examined, it would appear that uniparental inheritance is characteristic of these ferns as well in *O.* ×*ruggii*. (Stein, 1985). A similar result has been found in a tree fern hybrid, *Cnemidaria horrida* (L.) K. Presl × *Cyathea arborea* (L.) Smith (Stein & Conant, unpublished). Such studies, which examine chloroplast DNAs from mature plants, do not rule out the possibility of chloroplast inheritance from both parents with subsequent sorting of the chloroplast types. However, in *Phyllitis scolopendrium* the mode of plastid transmission is apparently biparental, yet both green and pale plastids were maintained (not sorted) in the mature sporophytes (Andersson-Kotto, 1931). Examination of very young hybrid individuals by Southern hybridization would address this question. Duckett & Bell (1971) reported that male gametes of archegoniate plants often do not transmit plastids to the egg. Thus, despite the one report of biparental transmission of chloroplasts in ferns (Gillham, 1978), uniparental, maternal inheritance of chloroplasts in ferns seems more likely to be a common pattern.

We also examined nuclear ribosomal DNA cut with eight restriction enzymes for evidence of the hybrid nature of these plants (Fig. 2). Seven of the eight endonucleases did not reveal informative restriction fragment length polymorphisms. However, the restriction enzyme *Apa* I cut the ribosomal DNA in the two parental types to yield several similar large fragments (ranging from 14.7 kb to 4.0 kb) and also two small fragments of slightly different mobility, 1.6 kb in *P. acrostichoides* and 1.4 kb in *P. braunii*. These small fragments are both present in the hybrids, as expected for nuclear DNA. A similar additivity of different lengths of ribosomal DNA fragments was demonstrated in an intergeneric hybrid between *Tolmiea menziesii* and *Tellima grandiflora* (Saxifragaceae) by Doyle et al. (1985). It is likely that these small fragments come from the intergenic spacer region, which has been shown to be the most variable (Appels & Dvorak, 1982), and length variation in this region may be related to loss or gain of a subrepeat. In addition to the ribosomal DNA fragment additivity, starch-gel electrophoresis revealed that all four hybrids summed isozyme bands fixed in each progenitor species at PGM-1 and SkDH (Barrington, unpublished) but did not show differences in fixed heterozygosity. Thus, while the isozymes and the ribosomal DNA data both verified that these ferns were hybrids, only the chloroplast DNA analysis demonstrated that they were undergoing recurrent formation.

The morphology of the hybrids was examined by one of us (DSB), while only the other (DBS) was aware of the genetic makeup of the cytoplasm. The 26 morphometric characters evaluated to determine if the cytoplasm accounted for a shift in morphology toward that of the parent contributing the cytoplasm included 21 leaf size and shape characters, stomate length and width, sorus position, receptacle shape, and true-indusium diameter. No clearcut relationship between morphology and the cytoplasmic compositions was detected in a discriminant function analysis of the hybrids and their parents.

The discovery of two chloroplast genomes in the population of hybrid *P.* ×*potteri* suggests that examination of a number of hybrid individuals might be routinely carried out as a means of documenting parentage and demonstrating recurrent hybrid formation. For example, Stein & Conant (unpub-

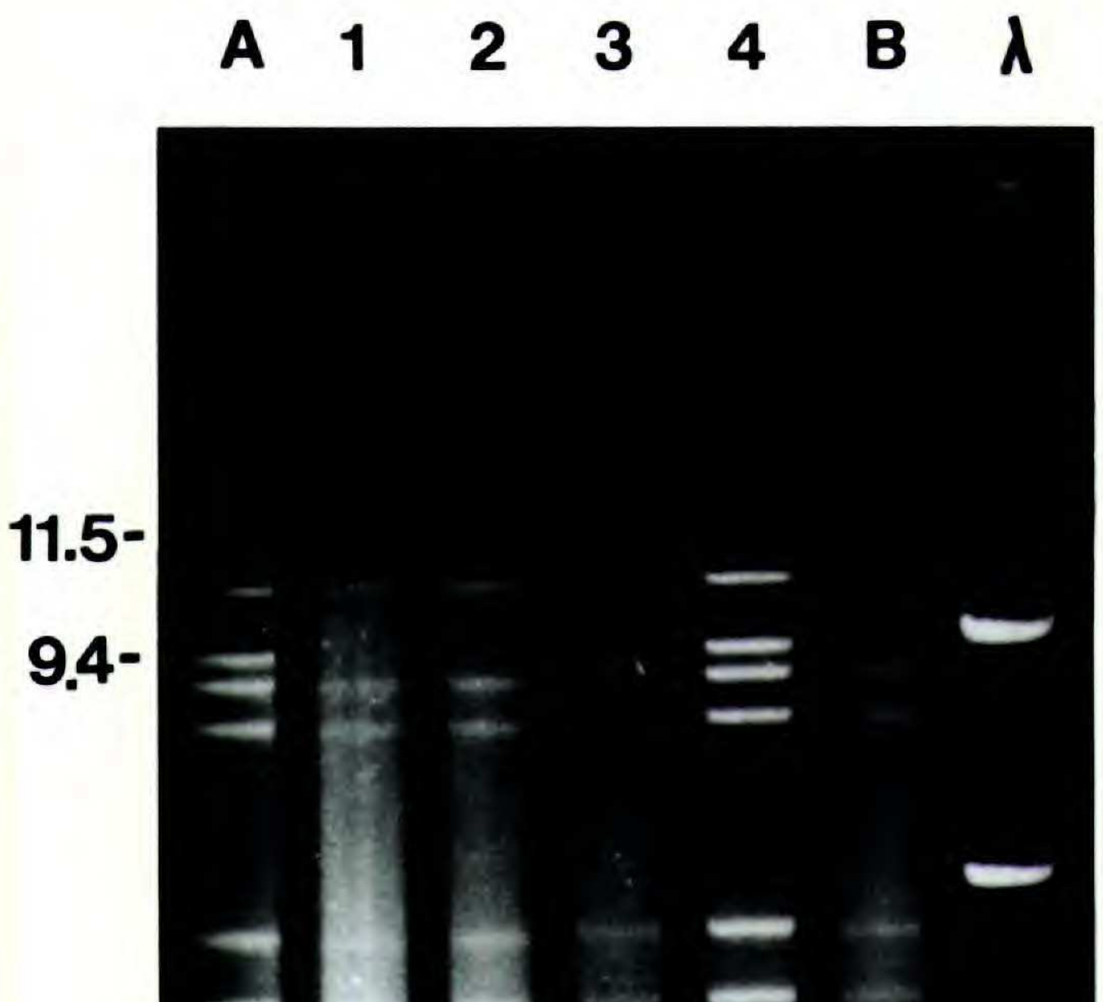

FIGURE 1. Chloroplast DNAs from *Polystichum acrostichoides* (A), *P. braunii* (B), and four hybrid plants of *P.* × *potteri* (1, 2, 3, 4) digested with *Hind* III. The fragments were separated on 0.7% agarose by gel electrophoresis. Hybrids 1 and 2 contain the *P. braunii* chloroplast genome; hybrids 3 and 4 have the *P. acrostichoides* chloroplast genome. λ = λ DNA cut with *Hind* III. The bands in this lane correspond to (from the top) 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb.

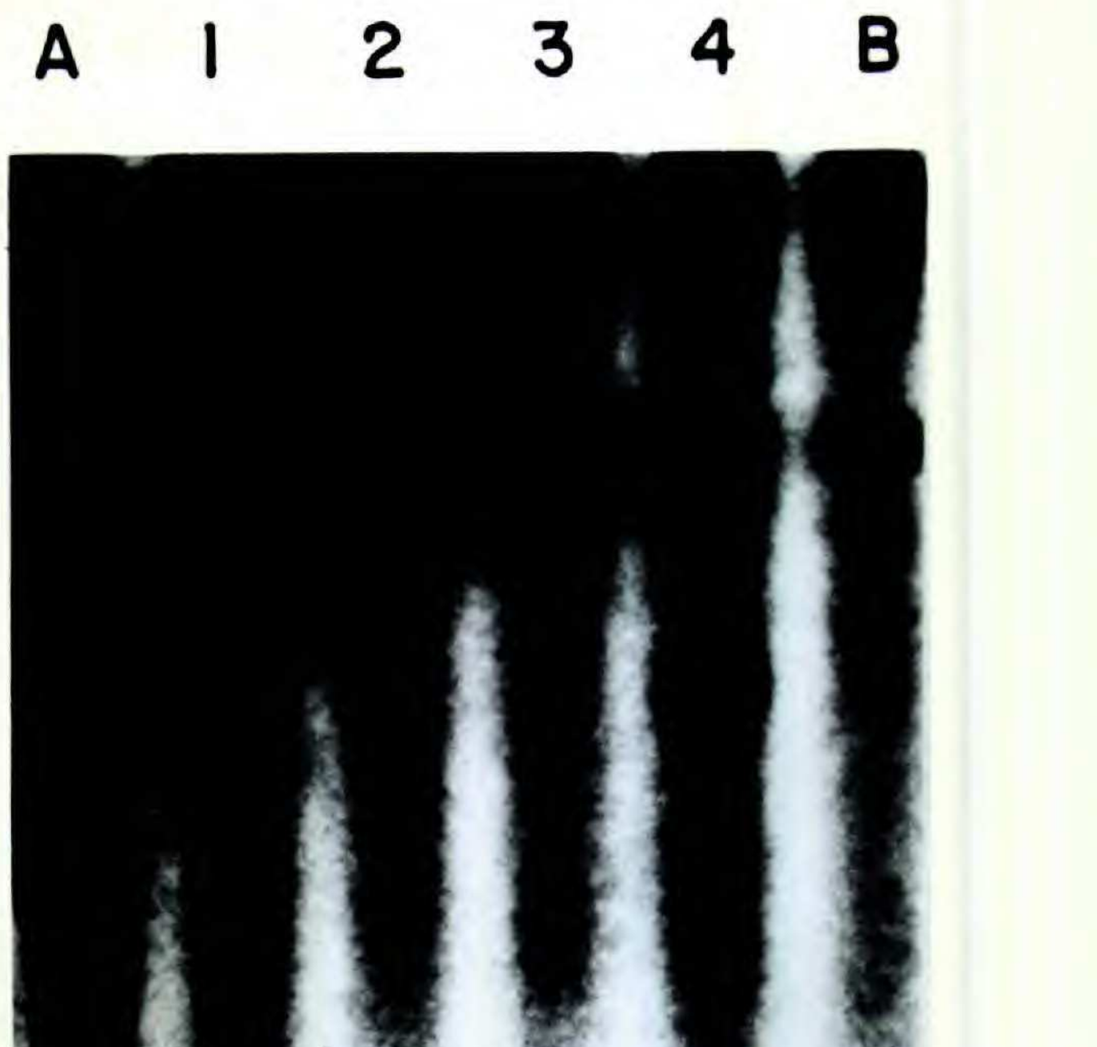

FIGURE 2. Ribosomal DNA fragment length polymorphisms in *P. acrostichoides* (A), *P. braunii* (B), and four hybrid plants of *P.* × *potteri* (1, 2, 3, 4) digested with *Apa* I. The fragments were separated on 0.7% agarose, transferred to Zetabind by Southern blotting, and hybridized to a ^{32}P-labeled ribosomal DNA repeat (pHA1). *Polystichum acrostichoides* and *P. braunii* contain 1.6 kb and 1.4 kb fragments, respectively; the hybrids contain both fragment lengths.

lished), in a preliminary examination of the tree ferns, examined nine individuals of *Cnemidaria horrida* × *Cyathea arborea* for chloroplast DNA composition. Eight contained the chloroplast DNA of *Cnemidaria horrida*; the ninth revealed the chloroplast genome of *C. arborea*. This documents the second parent; but more importantly it shows that hybridization has occurred more than once in these tree fern hybrids.

Chloroplast DNA has also been examined in studies of polyploid species in other evolutionary groups. Bryophyte allopolyploids have been shown to have had multiple origins. In a study of *Plagiomnium medium* (Bruch, Schimper & Gumbel) Kop. (Wyatt et al., 1988), an analysis of isozymes and chloroplast DNA documented allopolyploidy and the maternal parent, respectively. Only one chloroplast DNA type was found, but the study does not state how many individuals were examined for their DNA composition.

Multiple origin of allopolyploids has recently been documented in several angiosperm taxa. R. Wallace & R. Jansen (in prep.) have used chloroplast DNA to demonstrate recurrent hybrid formation in *Microseris* (Asteraceae). An allotetraploid, *M.*

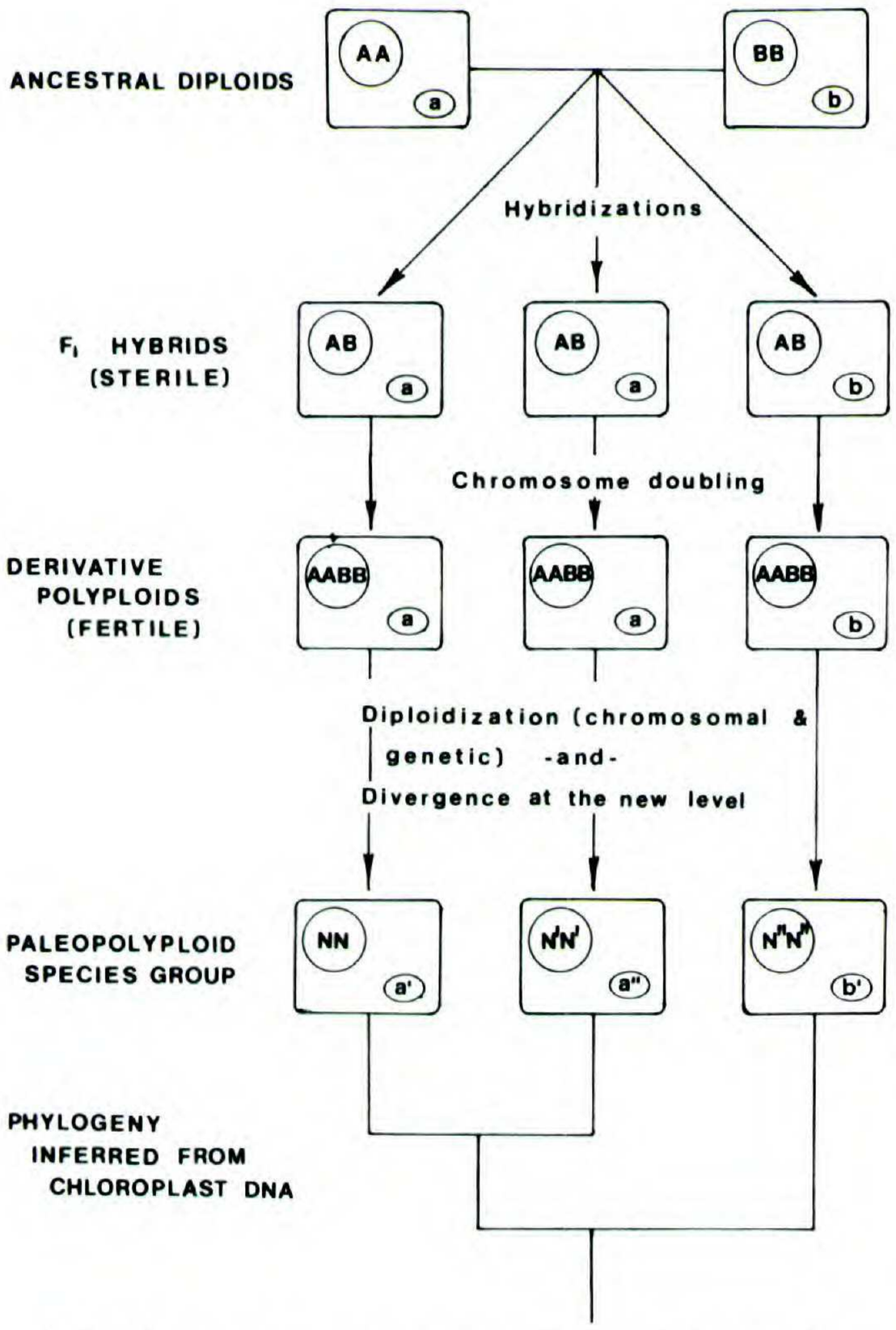

FIGURE 3. A hypothetical evolutionary history showing formation of paleoallopolyploids that subsequently gave rise to three modern species. In this case two of the modern species would contain chloroplasts derived from one chloroplast genome (e.g., the "a" chloroplast genome), whereas one of the modern species would have chloroplasts derived from a different chloroplast genome (e.g., the "b" chloroplast genome). Thus, relationships as measured by nuclear DNA would suggest equal divergence, whereas chloroplast DNA would support a closer relationship between two of the three descendants.

heterocarpa (Nutt.) K. Chambers, has originated twice. During each origin, the chloroplasts were contributed by different subspecies of *M. douglasii* (DC.) Schultz-Bip. (i.e., subsp. *douglasii* and subsp. *tenella* [Asa Gray] Chambers). No tetraploid so far examined has contained the chloroplast genome of *M. lindleyi* (DC.) A. Gray, the other progenitor of the tetraploid. Similarly, Doyle, Doyle, Brown, and Grace (pers. comm.) have provided evidence of multiple origin of allotetraploid *Glycine tabacina* (Labill.) Benth., taking advantage of cpDNA variation within one of the progenitors. In a cpDNA study of the *Tragopogon* polyploid complex, Soltis & Soltis (1989) found that the cpDNA genomes of the two progenitors (*T. dubium* Scop. and *T. pratensis* L.) are each represented in different populations of their allotetraploid *T. miscellus* Ownbey.

The fact that two chloroplast genomes exist in current populations of hybrids and allopolyploid species suggests that paleopolyploids (ancient polyploids whose progenitors are extinct) would have a high probability of having similar cryptic variation. Thus, measuring relationships with cpDNA comparisons could give differing patterns of relationship if two species were descended from tetraploids of similar ancestry but carrying different chloroplast genomes. Figure 3 illustrates a set of events that would yield such a spurious phylogeny. While such a scenario might be rare, it may be a useful model to consider when chromosome numbers suggest ancient polyploidy, as for example in the fern genus *Osmunda* (Wagner & Wagner, 1980).

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FELIX QUI POTUIT RERUM COGNOSCERE CAUSAS (VIRGIL).
(Photo taken by Walter H. Hodge.)